Introduction to phage biology and phage display

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Introduction

Display of peptides and proteins on filamentous phage—phage display—is an *in vitro* selection technique that enables polypeptides with desired properties to be extracted from a large collection of variants. A gene of interest is fused to that of a phage coat protein, resulting in phage particles that display the encoded protein and contain its gene, providing a direct link between phenotype and genotype. This allows phage libraries to be subjected to a selection step (e.g. affinity chromatography), and recovered clones to be identified by sequencing and re-grown for further rounds of selection. Since the initial description of the approach by Smith (1), it has become established as a powerful method for identifying polypeptides with novel properties, and altering the properties of existing ones (for reviews, see 2–5).

Filamentous phages are ideal in many ways for use as cloning vehicles and for display in particular. The genome is small and tolerates insertions into non-essential regions; cloning and library construction are facilitated by the ability to isolate both single- and double-stranded DNA (ssDNA and dsDNA), and by the availability of simple plasmid-based vectors; coat proteins can be modified with retention of infectivity; phage can accumulate to high titers since their production does not kill cells; and phage particles are stable to a broad range of potential selection conditions.

This chapter is intended to provide the background needed to initiate a phage display project. In the first portion (Sections 2 and 3), the life cycle, genetics, and

structural biology of filamentous phages are summarized, with a focus on aspects that are relevant to phage display. We then go on to describe general considerations to be made when approaching a new phage display project, including choice of display format, experimental design, and common pitfalls (Sections 4–6). Finally, summaries of commercial sources of phage display vectors, kits, and alternative display systems are provided for those cases in which such reagents can provide a head-start for investigators (Sections 7 and 8). Cross-references are provided to later chapters in the book that provide detailed procedures.

Biology of filamentous phage

Introduction

Filamentous phages constitute a large family of bacterial viruses that infect many gram-negative bacteria. Their defining characteristic is a circular, ssDNA genome encased in a long, somewhat flexible tube composed of thousands of copies of a single major coat protein, with a few minor proteins at the tips. The genome is small—a dozen or fewer closely packed genes and an intergenic (IG) region that contains sequences necessary for DNA replication and encapsidation. Unlike most bacterial viruses, filamentous phages are produced and secreted from infected bacteria without cell killing or lysis (*Figure 1*). Readers are referred to several excellent reviews on filamentous phage (6–9) for more comprehensive information and citations of the primary literature than are given here.

Most information about filamentous phages derives from those that infect *E. coli*: f1/M13/fd, and to a lesser extent IKe. These phages are characterized by a fivefold rotation axis combined with a twofold screw axis. Phages f1, M13, and fd are those that have been used for display. Their genomes are more than 98% identical and their gene products interchangeable, and the phages are usually referred to collectively, as Ff phages. Unless specified, the properties of filamentous phage described below refer to them together.

Structure of the phage particle

Filamentous phages have a fixed diameter of about 6.5 nm and a length determined by the size of their genome. The 6400-nucleotide ssDNA of Ff is encapsidated in a 930 nm particle, while a 221-nucleotide "microphage" variant is 50 nm long (10). Cloning DNA into a nonessential region of the genome can create longer phage, although the longer they are, the more sensitive the particles are to breakage (e.g. from vortexing).

Phage particles are composed of five coat proteins (*Figure 2*). The hollow tube that surrounds the ssDNA is composed of several thousand copies of the 50-residue major coat protein, pVIII, oriented at a 20° angle from the particle axis and overlapped like fish scales to form a right-handed helix (8). The filament is held together by interactions between the hydrophobic midsections of adjacent subunits. Except for five surface-exposed N-terminal residues, pVIII forms a single, continuous α-helix. The four positively charged residues near

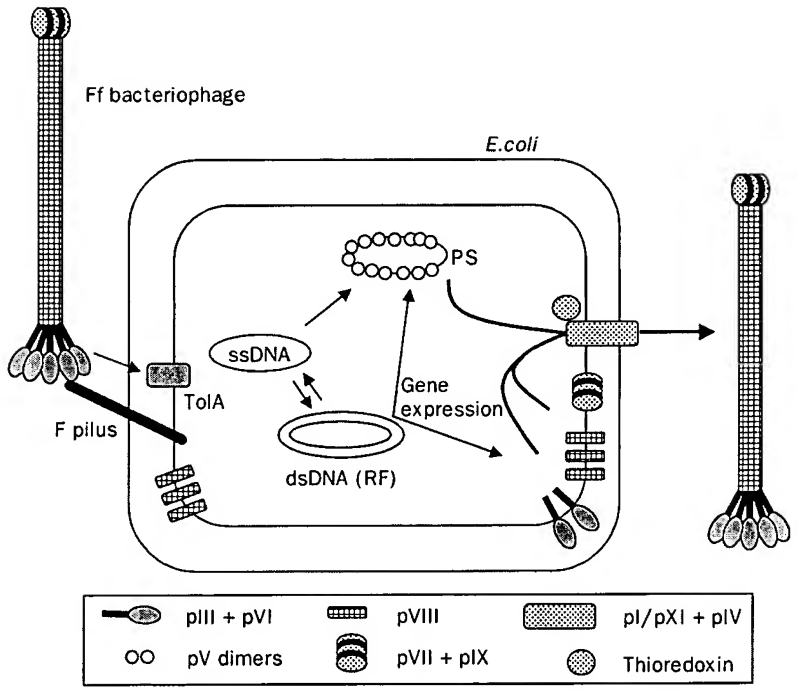

Figure 1 Life cycle of filamentous phage f1 (M13/fd). Sequential binding of pIII to the tip of the F-pilus and then the host Tol protein complex results in depolymerization of the phage coat proteins, their deposition in the cytoplasmic membrane (where they are available for re-utilization), and entry of the ssDNA into the cytoplasm. The ssDNA is converted by host enzymes to a double-stranded RF, the template for phage gene expression. Progeny ssDNA, coated by pV dimers (except for the packaging sequence hairpin (PS) that protrudes from one end), is the precursor of the virion. A multimeric complex that spans both membranes—composed of pI, pXI, pIV, and the cytoplasmic host protein thioredoxin—mediates conversion of the pV–ssDNA complex to virions and secretion of virions from the cell. This process involves removal of pV dimers and their replacement by the five coat proteins that transiently reside in the cytoplasmic membrane.

the C-terminus of pVIII are at the inner surface of the tube and interact with phosphates of the viral ssDNA.

The ends of the particle are distinguishable in electron micrographs. The blunt end contains several (3–5) copies each of pVII and pIX, two of the smallest ribosomally translated proteins known (33 and 32 residues, respectively). Neither their structure nor disposition in the particle is known. However, immunological evidence indicates that at least some of pIX is exposed (11) and antibody variable regions have been successfully displayed on the amino termini of pVII and pIX (12). Phage assembly begins at the pVII–pIX end, and in the absence of either protein, no particle is formed.

The pointed end of the particle contains about five copies each of pIII and pVI, both of which are needed in order for the phage to detach from the cell membrane; pVI is degraded in cells that lack pIII, which suggests that these proteins

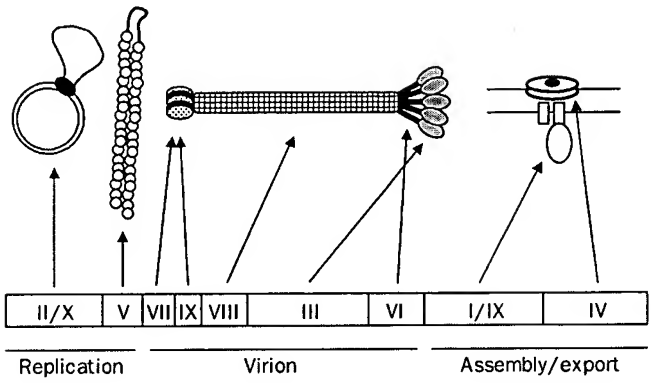

Figure 2 Filamentous phage f1 (M13/fd) genes and gene products. *Gene II* encodes pII, which binds in the IG region (located between genes IV and II/X; not shown) of dsDNA and makes a nick in the + strand, initiating replication by host proteins. pX is required later in infection for the switch to ssDNA accumulation. *Gene V* encodes the ssDNA binding protein pV. *Genes VII* and *IX* encode two small proteins located at the tip of the virus that is first to emerge from the cell during assembly. *Gene VIII* encodes the major coat protein, and *genes III* and *VI* encode pIII and pVI, which are located at the end of the virion and mediate termination of assembly, release of the virion, and infection. *Gene I* encodes two required cytoplasmic membrane proteins, pI and pXI, and *gene IV* encodes pIV, a multimeric outer membrane channel through which the phage exits the bacterium. Note that the genome is in fact circular, but is shown in a linear presentation here for clarity.

assemble in the cell membrane before their incorporation into phage particles (13). They can be isolated from phage as a complex (14). The disposition of pVI in the particle is not known, but pVI with fusions to the C-terminus can be incorporated into phage, suggesting that this portion of the 112-residue pVI may be surface exposed (13).

More is known about the 406-residue pIII, the most commonly used coat protein for display (*Figure* 3). Its N-terminal domain, which is necessary for phage infectivity, is surface exposed and forms the small "knobs" that can often be seen to emanate from the pointed end of the particle in electron micrographs. Three pIII domains have been defined, the two N-terminal of which (N1 and N2) are believed to interact intramolecularly, based on crystallographic analysis (16, 17). The three domains are separated by two long, presumably flexible linkers characterized by repeats of a glycine-rich sequence. The final 132 residues within the C-terminal CT domain are necessary and sufficient for pIII to be incorporated into the phage particle and to mediate termination of assembly and release of phage from the cell; this domain is likely to be buried within the particle (13).

The single-stranded phage genome is oriented within the phage particle. Its orientation is determined by the packaging signal (PS), located in the non-coding IG region of the genome. The PS, an imperfect but extremely stable hairpin, is positioned at the pVII–pIX end of the particle and is necessary and sufficient for efficient encapsidation of circular ssDNA into phage particles. Certain amino acid

substitutions in pVII, pIX, and pI (see below) enable single strands that lack a PS to be encapsidated; it is not known whether the DNA is randomly oriented in such particles or if some small duplex region serves as a secondary PS.

Infection

All filamentous phages that have been characterized use pili, which are long and slender cell surface appendages that resemble the phage themselves, as receptors. There are many different kinds of pili. *E. coli* phage use self-transmissible pili that mediate transfer of the plasmid that encodes them to recipient bacteria. Ff phage bind F pili, and IKe uses N and P pili. Phage can infect cells that lack appropriate pili, but the process is extremely inefficient; the efficiency is improved two to four orders of magnitude by agents that concentrate the phage or promote its adherence to the cell surface, such as $CaCl_2$ and polyethylene glycol (18).

Infection normally begins when the N2 domain of pIII (*Figure 3*) binds to the tip of a pilus (19) (see *Figure 4*). As might be expected from the low abundance of F pili (only a few per cell) and the small target size that their ends present, the rate and efficiency of infection of a bacterial culture is improved at high multiplicity of phage per cell and high cell density. However, if high density is achieved by growing cells past log phase (as opposed to concentrating them gently), pilus expression is decreased and infectivity compromised. Furthermore, since F pili do not assemble at low temperatures, efficient infection (and therefore plaque formation) by Ff requires incubation at or above 34 °C.

Pili normally assemble and disassemble continuously, and this, possibly stimulated by phage binding, brings the phage close to the cell surface. Upon pilus binding to N2, the N1 domain is released from its normal interaction with N2, making it available to bind to the host TolA protein, which extends into the periplasm from the cytoplasmic membrane (20, 21). Thus, the infection process appears to conform to a classic model involving a receptor (F-pilus) and a co-receptor (TolA). How the phage penetrates the outer membrane and the

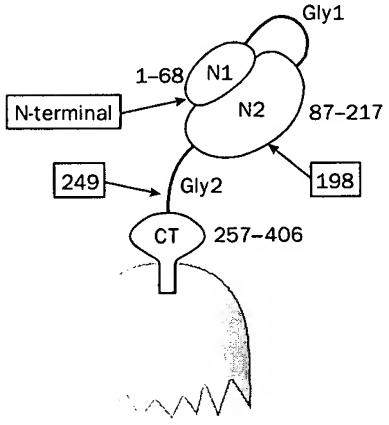

Figure 3 Domain structure of pIII and fusion points for display. The three domains of pIII are shown: from N- to C-terminus, the N1, N2, and CT (C-terminal) domains are each separated by glycine-rich linker regions (Gly1 and Gly2). CT is buried within the phage particle and is required for virion assembly. Three positions at which display fusions are commonly created are indicated (boxed); numbers refer to residues in the mature protein sequence. Note that fusion at position 198 (corresponding to a convenient *Bam*HI restriction site) leaves an unpaired cysteine residue in the N2 domain (C201) that can interfere with display (15).

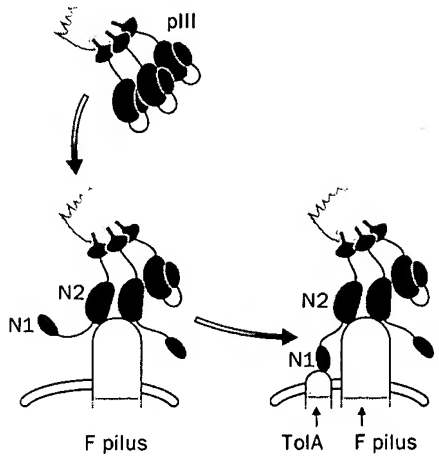

Figure 4 Infection of *E. coli* by Ff bacteriophage. Infection is initiated by interaction of the N2 domain of pIII with an F pilus projecting from an *E. coli* cell. This interaction releases the N1 domain from its intramolecular interaction with N2, allowing it to bind to a discrete domain (D3) of the bacterial TolA protein. Subsequent steps are not yet characterized. The bacterial outer membrane is omitted from the diagram for clarity—it is not yet clear how infecting phage particles penetrate this membrane.

underlying peptidoglycan layer is not known. Three Tol proteins (Q, R, and A), all integral cytoplasmic membrane proteins, are absolutely required for phage infection (22, 23). They mediate depolymerization of the phage coat proteins into the cytoplasmic membrane and the translocation of the viral ssDNA into the bacterial cytoplasm, although the molecular details of how this is accomplished remain to be determined.

Replication

Upon entry of the viral single-stranded phage DNA (the + strand, which has the same polarity as the mRNA), host RNA and DNA polymerases and topoisomerase convert it to a double-stranded, super-coiled molecule called Replicative Form (RF; see *Figure 1*). The RF serves as template for phage gene expression, and this expression—in particular, synthesis of pII, a site-specific nicking-closing enzyme—is necessary for further replication. Through a rolling circle mechanism, pII nicks the + strand of the RF at a specific site in the non-coding IG region of the phage genome, and the 3′ end of the nick is elongated by host DNA polymerase III using the – strand as template. The original + strand is displaced by Rep helicase as the new + strand is synthesized, and when a round of replication is complete, the displaced + strand is recircularized by the nicking-closing activity of pII and again converted to RF. Synthesis of the – strand requires an RNA primer. The primer is generated by RNA polymerase, which initiates synthesis at an unusual site in the IG region of the + strand consisting of two adjacent hairpins that include promoter-like −35 and −10 motifs separated by a single-stranded region (24).

During the early phase of infection, when the concentration of the phage ssDNA-binding protein (pV) is low, newly synthesized single strands are immediately converted to RF, and both RF and phage proteins increase exponentially. As its concentration increases, pV binds cooperatively to newly

generated + strands (*Figure 1*), preventing polymerase access and blocking their conversion to RF. The restart protein pX, which is identical to the carboxy-terminal 111 residues of pII, is required for the stable accumulation of single strands at this stage, but the mechanism by which it acts is not known (25). pV is dimeric, with the interaction surface of the subunits opposite the DNA-binding surface. Thus upon binding, the back-to-back arrangement of the dimers collapses the circular single strand into a rod-like structure. The DNA is oriented in the complex, with the PS hairpin protruding from one end, presumably because pV bind dsDNA only weakly. The pV/ssDNA complex is the substrate for phage assembly.

Genes and gene expression

The ~6400 nucleotide Ff genome contains nine closely packed genes (*Table 1*), and one major non-coding region (the IG) which contains the replication origins for + and – strand synthesis and the PS. Two of the phage genes (I and II) have internal translational initiation sites from which in-frame restart proteins are produced. In each case, both the full-length and the restart protein (whose sequence is identical to the carboxy terminal third of the full-length protein) are necessary for successful phage production. Of the 11 phage-encoded proteins, three (pII, pX, pV) are required to generate ssDNA, three (pI, pXI, pIV) are required for phage assembly, and five (pIII, pVI, pVII, pVIII, pIX) are components of the phage particle (*Figure 2*).

All phage proteins are synthesized simultaneously, although diverse mechanisms ensure that each is produced at an appropriate rate. There are differences in promoter and ribosome binding site strength or accessibility. A weak rho-dependent termination signal at the beginning of gene I limits its transcription, and the large number of infrequently used codons reduces its rate of translation. At the other end of the spectrum, overlapping transcripts from multiple

Table 1 F-specific filamentous phage genes/proteins and properties

Gene	Protein	Size (aa)	Function	Location	Used for display?
I	I	348	Assembly	Inner membrane	
	XI	108	Assembly	Inner membrane	
II	II	409	Replication (nickase)	Cytoplasm	
	X	111	Replication	Cytoplasm	
III	III	406[a]	Virion component	Virion tip (end)	Yes (N-term)
IV	IV	405[a]	Assembly (exit channel)	Outer membrane	
V	V	87	Replication (ssDNA bp)	Cytoplasm	
VI	VI	112	Virion component	Virion tip (end)	Yes (C-term)
VII	VII	33	Virion component	Virion tip (start)	Yes (N-term)
VIII	VIII	50*	Virion component	Virion filament	Yes (N- and C-term)
IX	IX	32	Virion component	Virion tip (start)	Yes (N-term)

[a] Mature protein without signal sequence.

promoters (there are only two terminators) and multiple RNA processing events increase the abundance of RNAs for the genes closest to the terminators (26). This results in high levels of pV and pVIII, the proteins that are required in the greatest quantities.

At later times after infection, the rates of phage protein and DNA synthesis taper off. This occurs when the high concentration of pV sequesters the + strands and prevents their conversion to RF, the template for gene expression. In addition, excess pV binds to a tetraplex structure in the gene II and gene X mRNAs (27), repressing their translation, and the reduction in pII levels leads to lower rates of + strand synthesis (25, 28). At these lower synthetic rates, a steady-state level of phage products is maintained by the secretion of progeny phage and by the continued growth and division of the infected cells, and phage production continues at a linear rate.

Physiology of phage assembly

The unique aspect of filamentous phage assembly is that it is a secretory process. Assembly occurs in the cytoplasmic membrane, and nascent phages are secreted from the cell as they assemble. All eight of the phage-encoded proteins that are directly involved in assembly are integral membrane proteins (*Figure 1*). This includes three non-virion proteins—pI and its restart partner, pXI, in the cytoplasmic membrane and pIV in the outer membrane—and the five viral coat proteins, which reside in the cytoplasmic membrane prior to their incorporation into phage. Two (pIII and pVIII) are synthesized as precursors and, after signal sequence cleavage, span the membrane with their C-termini in the cytoplasm. The orientation of the other coat proteins is not known with certainty. pVI is particularly hydrophobic (57% of its residues are hydrophobic) and could span the membrane more than once.

The first progeny phage particles appear in the culture supernatant about 10 min after infection (at 37 °C). Their numbers increase exponentially for about 40 min, after which the rate becomes linear. About 1000 phage per cell are produced in the first hour. Under optimal conditions, the infected cells can continue to grow and divide—and produce phage—indefinitely. Persistent infection is possible because the five capsid proteins and the viral single strands are removed from the cell (by assembly and secretion) at a rate commensurate with their synthesis, and over-accumulation of the non-secreted proteins is prevented by the pV/pII regulatory loop and by dilution resulting from continued cell growth and division. The continued growth of phage-producing cells (at a rate significantly lower than uninfected bacteria) accounts for the turbidity of Ff plaques. When the plaques are clear rather than turbid, the infected cells are being killed. This occurs when there is an imbalance between component synthesis and phage assembly. For example, wild type Ff phage form clear plaques at 39 °C (and very small plaques at 42 °C) because the intrinsic temperature-sensitivity of pV results in reduced accumulation of ssDNA and thus a lower rate of phage production. Even modest perturbations of the phage life cycle can lead to the eventual death of infected cells. Phage into which additional DNA has been

cloned often give rise to smaller and/or clearer plaques; this may be due to the imbalance caused by the increased utilization of major compared to minor coat proteins in such particles.

The mechanics of phage assembly

Phage assembly can be divided into five stages: preinitiation, initiation, elongation, pretermination, and termination. Preinitiation is defined as the formation of an assembly site, a region visible by electron microscopy where the cytoplasmic and outer membranes are in close contact (29). Assembly sites are composed of the three morphogenetic proteins, pI, pXI, and pIV, which interact via their periplasmic domains (N-terminal for pIV and C-terminal for pI and, presumably, pXI); the sites form independently of any other phage proteins.

pIV is a cylindrical structure with a central cavity about 8 nm in diameter; it is composed of 12–14 identical subunits with a combined mass of about 600 kDa (30). In the cryo-EM structure of pIV (MR *et al.*, unpublished data), there is some density within the cavity, which presumably explains why the normal state of the pIV channel is closed. Certain mutant forms of pIV, however, open frequently and allow entry of foreign substances into the bacterial periplasm (31, 32). Phage that lack pIII and are thus unable to be released from the cell block such entry, indicating that phage do pass through the pIV channel (33). Nothing is known about the interior of the pIV channel, except that it can accommodate heterologous pVIII from IKe (34), as well as an occasional pVIII subunit carrying a large N-terminal extension or pVIII uniformly substituted with short (6–8 residue) N-terminal extensions, as in phage display (35, 36). The dimensions and/or properties of the channel may be a factor limiting the size of polypeptides that can be displayed on pVIII.

pI and pXI also form a multimeric complex composed of about 5–6 copies of each; its shape and dimensions are not known (J. N. Feng, P. Model and M.R., unpublished data). In the absence of the other phage proteins, pI/pXI causes membrane depolarization, which suggests that the complex may also be a channel. Thus the assembly site may be an extremely large channel that traverses both bacterial membranes. The cytoplasmic N-terminal domain of pI (absent from pXI) contains a conserved nucleotide-binding motif; since this motif is essential for phage assembly (37), and phage assembly requires ATP hydrolysis (38), pI is likely to be an ATPase.

Initiation takes place only if the assembly site, the two minor coat proteins (pVII and pIX) located at the beginning tip of the particle, and the ssDNA substrate are present (37). Genetic analyses suggest that pVII and pIX (in the membrane) interact with the PS, which protrudes from one end of the pV-ssDNA complex (in the cytoplasm), and that the PS associates with the cytoplasmic domain of pI. Host-encoded thioredoxin, a small, cytoplasmic protein known as a potent reductant of protein disulfides, also interacts with pI. Although phage assembly does not utilize this redox activity, thioredoxin appears to be part of the initiation complex, and may confer processivity to the elongation reaction.

Elongation involves the successive replacement of pV dimers that cover the viral DNA by membrane-embedded pVIII and translocation of the DNA across the membrane. The process continues until the end of the viral DNA has been coated by pVIII. If either pIII or pVI is absent, the largely extracellular phage particle remains tethered to the cytoplasmic membrane where it remains competent to resume elongation when another pV-ssDNA complex enters the assembly site; ultimately, tethered phage filaments of more than 10 times unit length accumulate (39). Even in normal infections when pIII and pVI are present, about 5% of progeny phage particles are double length. Such secondary rounds of elongation do not require reinitiation—ssDNA without a PS can be efficiently incorporated (40).

Pretermination (13) is the incorporation of the membrane-embedded pIII-pVI complex at the terminal end of the nascent phage particle. A fragment containing only the C-terminal 83 residues of pIII is sufficient to mediate this step, but cannot effect detachment of the phage from the cell. Termination or release of the phage, which requires a 93 residue C-terminal segment of pIII, has been proposed to consist of a conformational change in the pIII-pVI complex that detaches the complex (and the phage) from the cytoplasmic membrane (13). A still longer portion of pIII (the 132 C-terminal residues) is required for the formation of stable virus particles.

Coat proteins used for display

All five capsid proteins have been used to display proteins or peptides, to varying degrees (*Table 1*). One report has described the fusion of antibody fragments to the amino termini of both pVII and pIX, which constitute the "start" end of the virion tip (12). The pVI protein, which interacts with pIII at the "end" virion tip, has also been used to display polypeptides through a carboxy-terminal fusion—until recently the only way to directly create fusions in this orientation ((41); see Chapter 12). C-terminal linkage is particularly desirable for display of polypeptides encoded by cDNA fragments, since the inclusion of the stop codon at the end of the cDNA will not prevent display. Hence, pVI display has proved effective for the expression cloning of protein-protein interactors from cDNA libraries, as described in Chapter 12. However, by far the most commonly used virion proteins for phage display are pVIII and pIII.

pVIII

pVIII, the major coat protein, is present in several thousand copies in phage particles. Sequences for display are typically inserted at the N-terminus, between the signal sequence and the beginning of the mature protein coding sequence. However, only short peptides sequences (6–8 residues) can be displayed on every copy of pVIII in a virion—larger sizes prevent packaging of the particles, probably because of the size restrictions of the pIV channel through which phage pass during extrusion (see Section 2.7). Display of larger polypeptides on pVIII

requires expression of the fusion protein from a phagemid vector, yielding hybrid virions bearing mainly wild-type pVIII (see Section 4.2).

An emerging trend (for pVIII display but potentially more generally) is the use of protein engineering to modify the protein to broaden applicability: engineered pVIII proteins have been described that permit the display of large polypeptides at high copy number (42), or the display of proteins fused at the C-terminus of the protein (43).

pIII

pIII, present in five copies at the "end" tip of the virion, is the protein of choice for most phage display fusions due to its tolerance for large insertions, its compatibility with monovalent display (see Section 4.3), and the wide availability of suitable vectors. Although pIII is more tolerant than pVIII to substantial insertions, infectivity of the resulting phage can be reduced, sometimes dramatically. As with pVIII, this can be overcome by using phagemid constructs, resulting in the production of hybrid virions that also bear wild-type pIII (see Section 4.2). Since such virions no longer rely on the infectivity of the pIII fusion protein, proteins can instead be fused to truncated pIIIs designed with the structure of the protein in mind (*Figure 3*). These can confer more efficient display, by reducing or eliminating proteolysis of the fusion protein, as well as reducing the size of the phagemid vector. Potential disadvantages include the possibility of sterically hindering access to the displayed protein. Fusions have been reported at pIII residue 198 (44), which deletes the N1 domain and some of N2, or to residue 249 (45), which deletes N1 and N2 and fuses the displayed protein to a short portion of the second glycine-rich linker. C-terminal pIII display through fusion to a linker at the C-terminus of the pIII is also possible (46).

Starting a phage display project

Feasibility of display

The first step in a phage display project is establishing that display of the polypeptide of interest is feasible. In the early days of phage display, there were worries that the limitations of virion extrusion and infection would restrict display to small peptides and proteins, and furthermore that only proteins that are normally extracellular would be suitable, since display involves secretion of the fusion protein into the oxidizing environment of the bacterial periplasm. Both concerns have proved false, and intracellular and extracellular proteins of a wide range of sizes and structures have been functionally displayed (see *Table 2*). Although some proteins may be recalcitrant to display due to individual properties (such as toxicity to *E. coli*, or interference with phage production), size alone does not appear to be a major factor, and display of most proteins should be feasible.

For proteins that are normally intracellular, precautions can be taken to try to preserve the native structure of the molecule—for example, addition of zinc

Table 2 Examples of proteins displayed on filamentous phage[a]

Protein displayed	Molecular weight (kDa)	Format	Reference
Secreted proteins			
Z-domain (Protein A)	6.5	pIII	(47)
Mustard trypsin inhibitor (MTI-2)	7	pIII	(48)
C5a	8	pIII (Fos–Jun)	(49, 50)
Insulin-like growth factor (IGF)-1	14.5	pIII	(51)
Lipocalins	17.5	pIII	(52)
hGH	22	pIII, pVIII, pVIII (C-terminal)	(42–44)
Trypsin	24	pIII, pVIII	(53)
Antibody scFv fragments	25	pIII, pVIII	[b](54)
Subtilisins	28	pIII, pVIII	(55, 56)
Insulin-like growth factor binding protein (IGFBP)-2	31	pIII	(57)
Peptide–β2m–MHC complex	41	pIII	(58)
Vascular endothelial growth factor (VEGF)	2 × 11.5	pIII	(59)
Antibody Fab fragments	2 × 25	pIII, pVIII	(60)
Alkaline phosphatase	2 × 60	pIII	(61)
Streptavidin	4 × 15	pVIII	(42)
Intracellular proteins			
WW domain	4	pIII	(62)
Src Homology 3 (SH3) domain	6.5	pIII	(63)
FRAP/mTOR FRB domain	9.5	pIII	[c]
Zif268 zinc finger	10	pIII	[d]64
Cytochrome b_{562}	11	pIII	(65)
FK506 binding protein (FKBP)12	12	pIII, T7 display	[c](66)
Glutathione S-transferase (GST)	2 × 25.5	pIII	(67)

[a] This table is intended to indicate the range of proteins that can be displayed, but is neither complete nor exhaustive. Additional examples can be found in (2).

[b] See Chapter 13 for additional references.

[c] T. Clackson, unpublished data.

[d] See Chapter 9 for additional references.

during preparation of phage displaying zinc finger proteins (see Chapter 9). For proteins with lone cysteine residues, such as FKBP12, selections can be performed in the presence of dithiothreitol (DTT) (to which phage particles are stable—see Section 5.2) to maintain a reducing environment (T.C., unpublished data). If necessary, displayed proteins can even be refolded prior to selection by exposure of the particles to denaturants followed by dialysis (68).

It has also proved feasible to display multi-subunit proteins, by a variety of methods (*Table 2*). For heterodimers, such as antibody Fab fragments, one chain

can be displayed and another provided as a soluble protein cosecreted into the periplasm (60). Homo-oligomeric proteins can also be displayed by relying on proteolysis of the displayed fusion protein to release sufficient soluble protein, or by invoking interactions between displayed proteins, either inter- or intra-phage (e.g. see 61).

Phage or phagemid vector?

Proteins can be displayed using vectors based on the natural Ff phage sequence—phage vectors—or using plasmid-based "phagemid" vectors that contain only the fusion protein gene, and no other phage genes (for a review, see 3). The two alternatives are illustrated in *Figure 5* for display on pIII, although similar principles apply for display on other proteins. In phage vectors, the heterologous sequence for display is inserted directly into the coding sequence for pIII or

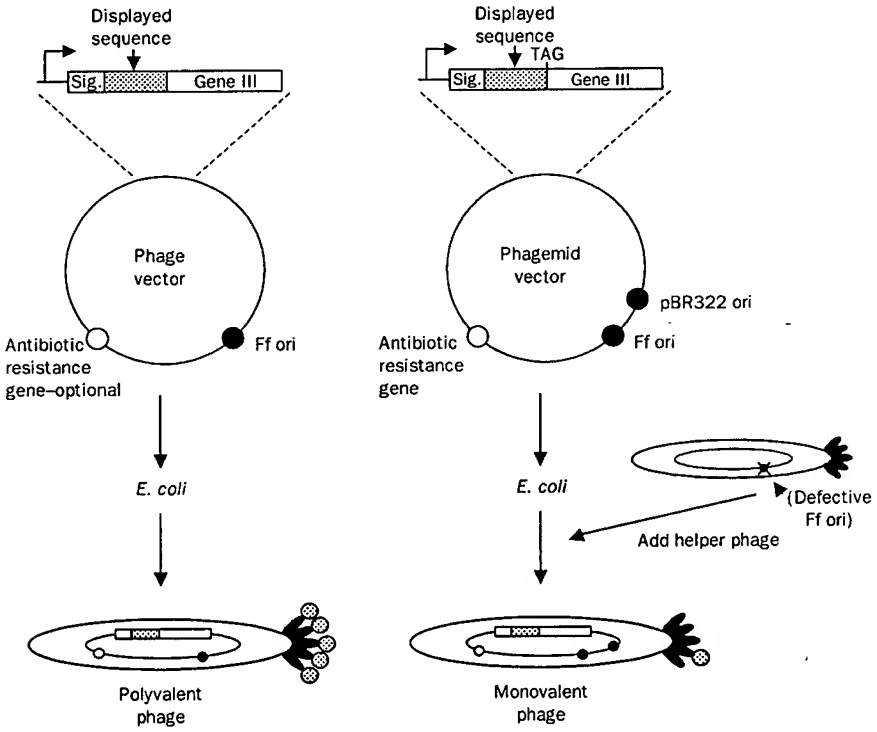

Figure 5 General scheme for phage display using phage or phagemid vectors. The difference between phage and phagemid vectors is illustrated for pIII display. Sequences for display are inserted between a secretion signal sequence ("sig.") and gene III. Both phage and phagemid vectors carry an Ff origin of replication to permit production of ssDNA and hence virions. Phagemid vectors also have a plasmid origin (here pBR322) and an antibiotic resistance marker to allow propagation as plasmids in *E. coli*. Phage vectors are also often modified with antibiotic resistance markers for convenience, as illustrated here. In many phagemid vectors, an amber stop codon (TAG) is interposed between the displayed sequence and gene III, to allow soluble protein expression by transferring the vector into a non-*supE* suppressor strain.

another coat protein. When introduced into *E. coli*, phage will be produced in which all copies of the coat protein display the heterologous protein (i.e. the protein is displayed polyvalently). Examples of pIII phage display vectors include the fUSE vectors constructed by Smith and coworkers (69), and the M13KE vectors commercially available from New England Biolabs (see Section 8).

In phagemid vectors, the displayed protein fusion gene is cloned into a small plasmid under the control of a weak promoter. In addition to a plasmid origin of replication, the vector also has an Ff origin to allow production of single-stranded vector and subsequent encapsidation into phage particles. To produce such particles, *E. coli* cells harboring the plasmid are infected with helper phage, which is an Ff phage with a compromised origin that leads to its inefficient packaging (see Section 4.4). The infected cells express all the wild-type phage proteins from the helper phage genome, as well as a small amount of the fusion protein encoded by the phagemid, so that phage particles are extruded by the cells that contain both proteins, usually with the wild-type in considerable excess. Because the helper phage genome is poorly packaged, nearly all the phage particles contain the phagemid genome, preserving the linkage between the displayed protein and its gene.

Phagemid vectors have been described for both pIII and pVIII display, although pIII is more common. pIII phagemid vectors are described in more detail in Chapter 4. A major advantage of phagemid vectors is their smaller size and ease of cloning, compared to the difficulties of cloning in phage vectors without disrupting the complex structure of overlapping genes, promoters, and terminators. This generally translates into much higher library sizes for phagemid vectors. In addition, the phagemid approach must be used if monovalent display is desired in order to obtain selection based on true binding affinity (see Section 4.3). For pVIII display, use of phagemids is generally required to achieve display of sequences longer than 6–8 amino acids. Phagemids have also been used for pVI display (see Chapter 12).

Polyvalent or monovalent display?

The choice of polyvalent or monovalent display is related to the choice of vector type. Conventional phage vectors with natural phage promoters will generally produce polyvalent display unless there is extensive proteolysis of the displayed proteins. In addition, phagemid vectors for pVIII display, in which only a fraction of the ~2700 copies are fusion proteins, will typically still display polyvalently. On the other hand, use of phagemid vectors to display protein on pIII under the control of a weak (or uninduced) promoter will typically lead to what is referred to as "monovalent display" (3, 44).

It is important to understand the nature of monovalent display. A typical preparation of phage particles prepared from *E. coli* harboring a pIII phagemid display vector and infected with helper phage will exhibit a Poisson distribution of fusion protein expression: 10% or less of the particles will display one copy of the fusion; a very small percentage will display two copies; and the remaining

majority of the particles will display only wild-type pIII. Thus, the major displaying species is monovalent, but most particles do not display at all.

The valency of display is important principally because of its impact on the ability to discriminate binders of differing affinities (see Chapter 4, Section 2.1). Early work (44) showed that polyvalent display prevented the highest-affinity clones in a selection from being identified, because multivalency conferred a high apparent affinity (avidity) on weak-binding clones. Monovalent display allows selection based on pure affinity, and is therefore generally preferred for the many studies where the aim is to identify the tightest binding variant(s) from a library. Conversely, in applications where the initial selectants are of very low affinity—for example, the *de novo* selection of peptides that bind a given target—polyvalency increases the chances of isolating rare and weakly binding clones. A frequent experimental strategy in such projects is to start with polyvalent display, and then move to monovalent display as the affinity of the displayed polypeptide matures (see Chapter 6).

Helper phage

A number of different helper phage for phagemid preparation have been developed and are commercially available, including R408 (70) (Stratagene), M13 K07 (71) (New England Biolabs, Amersham Pharmacia Biotech), and VCSM13 (Stratagene). In general, they can be used interchangeably. All have mutations that reduce packaging efficiency (to ensure phagemid genomes are preferentially packaged), and mutations that allow productive infection of bacteria harboring plasmids with Ff origins of replication ("interference-resistance"). M13 K07 and its derivative VCSM13 carry a kanamycin resistance gene to allow antibiotic selection of helper-infected cells. A procedure for preparing a helper phage stock starting with a commercial sample is provided in Chapter 2, *Protocol 6*.

General protocols for phage preparation and quantitation

Most phage display projects involve the basic procedures of preparing stocks of phage or phagemid particles from infected bacteria (propagation), and titering those stocks to determine the concentration of infectious particles. For phage vectors (and helper phage), determining the concentration of viable particles involves counting phage plaques on a lawn of host bacteria. A general procedure for propagation and titering of phage stocks is provided in *Protocol 1*. It is advisable to follow this procedure for plaque isolation whenever propagating phage vectors or helpers in order to ensure that a pure culture of viable phage is obtained.

For phagemid vectors, titration of phagemids involves infection of bacteria and counting of resulting antibiotic-resistant colonies. A simple procedure for titration of phagemid particles is given in Chapter 4, *Protocol 5*, and a procedure for phagemid propagation is given in Chapter 4, *Protocol 1*.

A convenient alternative to determine the infectivity of phage or phagemid preparations is estimation of concentrations by absorbance. The extinction

coefficient depends on the size of phage particles, which in turn depends on the size of the genome (see Section 2.2). In general, for a helper phage such as VCSM13, 1 $OD_{270} = 5 \times 10^{12}$ particles/ml, and for a 5 kb phagemid, 1 $OD_{270} = 1.1 \times 10^{13}$ particles/ml. However, calculations based on absorbance may overestimate the concentration of viable, infective particles.

Protocol 1

Titering and propagating plaque-purified filamentous phage[a]

Equipment and reagents

- Phage stock solution to be titered (i.e. an appropriate phage vector, or VCSM13 helper phage (Stratagene))
- 2YT media (10 g bacto-yeast extract, 16 g bacto-tryptone, 5 g NaCl; add water to 1 L, pH 7.0 with NaOH; autoclave and cool)
- 2YT /tet media (2YT, 15 μg/ml tetracycline)
- *E. coli* XL1-Blue stock (Stratagene; Tet^R)
- Luria–Bertani (LB) agar plates (5 g bacto-yeast extract, 10 g bacto-tryptone, 10 g NaCl; add water to 1 L, pH 7.0 with NaOH, 15 g agar; autoclave and cool to 55 °C; pour into petri plates)
- 2YT top agar (16 g bacto-tryptone; 10 g bacto-yeast extract; 5 g NaCl; 7.5 g agar; add water to 1 L. Heat to dissolve the agar and autoclave.)
- Sterile 10 ml culture tubes
- Sterile Pasteur pipettes (for propagation)
- Microwave
- 45 °C incubator or water bath (optional)

Method

A. Titration and plaque purification

1 If the LB agar plates are stored at 4 °C, prewarm them to room temperature. In this case it may also be helpful to allow them to dry overnight with lids in place.

2 Pick a single colony of XL1-Blue cells into 5–25 ml of 2YT/tet media, and incubate the culture at 37 °C with shaking or rotation to a density of about 1 OD_{550}.

3 Perform 10-fold serial dilutions of the phage stock in duplicate in 0.1 ml aliquots of 2YT media to yield estimated concentrations of 10^3–10^5 phage/mL.[b]

4 Melt the 2YT top agar (at least 3 ml per phage dilution) by microwaving, and cool to 45 °C, for example in a water bath or incubator.

5 For each phage dilution, mix 0.01 ml of phage with 0.5 ml of fresh XL1-Blue cells in a 10 ml culture tube.

Protocol 1 continued

6 To each tube, add 3 ml of top agar, rolling the tube quickly to mix.

7 Immediately pour the top agar mixture onto the surface of an LB agar plate, rotating the plate to spread the top agar quickly and evenly across the surface. If the top agar is too cool, lumps will form, making plaques difficult to count.

8 Allow the top agar to solidify, then incubate the plates at 37 °C overnight.

9 Plaques should appear as relatively clear discs against a background lawn of cells.

10 Calculate the phage concentration in the original stock solution (plaque-forming units/ml; pfu/ml) by multiplying the number of plaques per plate by 100 (because 0.01 ml of diluted phage is used per plate) and by the appropriate serial dilution factor.

B. Progagation

1 To propagate phage, grow fresh XL1-Blue cells as above, and pick a single isolated plaque using a Pasteur pipette by placing a finger over the open pipette end after inserting it through a plaque into the agar.

2 Using a pipette bulb, force the agar plug into 1 mL of fresh XL1-Blue cells.

3. Incubate for 1 h at 37° with shaking or rotation.

4. Dilute the cells into 25–1000 ml of 2YT media containing an appropriate antibiotic (e.g. 10 μg/ml kanamycin for M13K07 or M13VCS helper phage).

5 Incubate the culture for 12–15 h at 37 °C with shaking. Phage may be harvested and purified as described in Chapter 2, *Protocol 6*.

[a] This procedure is a modification of that described by Sambrook *et al.* (72) for plating M13 phage.

[b] A typical yield from XL1-Blue phage cultures grown in 2YT would be 10^{11}–10^{12} phage/ml.

General principles of a phage display project

The structure of a generic phage display project is depicted in *Figure 6*. In general, the procedure can be divided into three stages: creating (or obtaining) a library of (poly)peptide variants; selection; and analysis of the selected clones. Figure 6 and the text below include cross-references to chapters in the book to illustrate the alternative approaches that can be taken at each stage.

Making a library

For proteins that have not previously been displayed, the first stage is to construct a display vector and confirm functional display. Following this, a wide variety of molecular biology techniques can be deployed to introduce diversity into the displayed protein. For targeted introduction of diversity, oligonucleotide-directed mutagenesis is a powerful and versatile technique, and

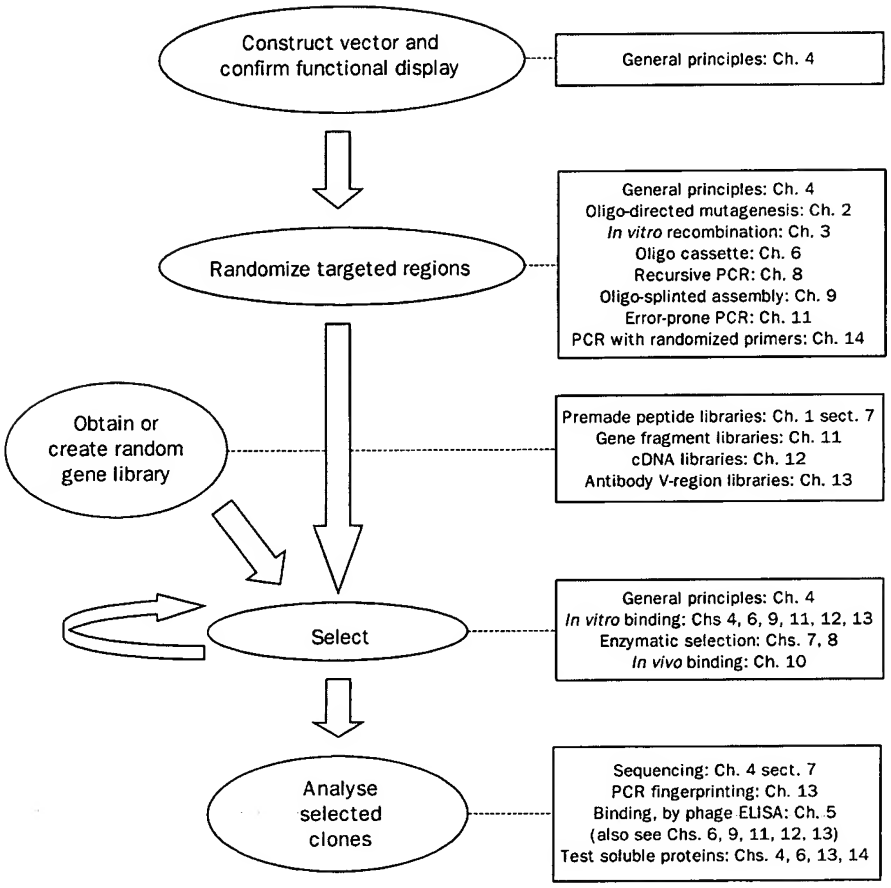

Figure 6 Flow diagram illustrating a generic phage display project, and examples of techniques that can be used at each stage (boxed).

recent advances have brought larger libraries of 10^{10}–10^{11} clones within easy reach (see Chapter 2). The diversity introduced can involve complete ("hard") randomization of residues, partial ("soft") randomization in which the wild-type residue is retained in some proportion, or "tailored" randomization, in which only a defined subset of amino acids is specified (see Chapter 2, Section 2, and Chapter 4, Section 3; for an example of tailored randomization, see Chapter 8). In all targeted libraries, it is important to bear in mind the implications of library size limitations on the total amount of diversity that can be surveyed in one library (see Chapter 2, Section 2). Alternatively, introduction of random changes throughout a sequence can be accomplished by *in vitro* recombination (Chapter 3). Numerous alternative approaches for introducing targeted diversity are described in other chapters throughout the book, as indicated in *Figure 6*.

Some specialized phage display applications involve the creation of libraries from collections of genes as opposed to diversifying a single gene. Examples

include cDNA libraries (Chapter 12) and antibody V-region libraries (Chapter 13). In these cases, specific protocols have been developed to create these libraries.

The creation of a library can be completely circumvented in some cases by obtaining an appropriate pre-made phage display library. Pre-made peptide display libraries are now commercially available (see Section 8).

Selection

The most common means of selection of desired clones is through an *in vitro* binding incubation, in which the phage library is bound to a target, washed, and then retained phage are eluted. This process is also referred to as "sorting" or "biopanning." The principles and alternatives for selections based on *in vitro* binding are described in detail in Chapter 4, Sections 4 and 5. A wide variety of washing and elution conditions have been used, exploiting the extraordinary stability of the Ff phage virion to extremes of pH, ionic strength, denaturants and even most proteases (with the exception of subtilisin) (see *Table 3*). This stability means that selection approaches are essentially limited only by the imagination of the investigator: for example, selection is even possible based on binding to targets *in vivo* after injection of phage libraries into whole animals (see Chapter 10).

The enrichment for binding clones over nonbinders conferred by a single round of selection can vary widely, from two-fold to more than a 1000-fold, although at least 10-fold is typical for an *in vitro* binding selection. In most cases, the eluted phage are used to reinfect *E. coli* for preparation of new phage ("amplified"), to allow further rounds of selection. Enrichment is typically monitored to help guide the decision to stop selecting and start analyzing clones.

Analysis of clones

In most phage display studies, one is interested in determining the identity of the clones that have been selected, and the properties of the displayed proteins. The most common technique for identifying selected clones is simply to sequence them. The principles of sequence analysis of phage-selected clones,

Table 3 Examples of conditions used for elution of target-bound phage

Elution buffer	Reference
0.1 N HCl, pH 2.2 with glycine; most common	(1)
20–100 mM HCl	(65, 73)
5–75 mM DTT (with reducable linker)	(55, 74, 75)
100 mM triethylamine	(76)
6 M urea, pH 3	(77)
50 mM sodium citrate, pH 2–6	(78)
500 mM KCl, 10 mM HCl, pH 2	(79)
4 M $MgCl_2$	(80)
Specific ligands	(45, 81)
Direct infection of *E. coli* host	(80, 82, 83)

and considerations for when to stop selecting and start analyzing, are described in Chapter 4, Section 7. An alternative and rapid approach for clone identification is PCR "fingerprinting," in which the insert encoding the displayed protein is amplified by PCR, and then digested with a frequent-cutting restriction enzyme (see Chapter 13, *Protocol* 19). Analysis of the digestion products gives a pattern of bands that may be unique to that clone. PCR fingerprinting techniques are particularly useful in applications such as cDNA library or phage antibody selections, where each clone is likely to have a different restriction pattern.

The most popular and versatile technique for initial characterization of the properties of selected clones, particularly for the majority of studies based on binding selections, is the phage ELISA. In this method, the target of interest is immobilized in wells of a 96-well plate, individual phage supernatants prepared from selected clones are added, and specific binding is detected by use of an anti-phage antibody. In addition to giving "yes–no" information as to whether a given clone binds the target, the assay can be used in a competition format to determine the relative binding affinities of clones, as described in Chapter 5.

Following characterization of the displayed polypeptide "*in situ*" on phage, the next step is often the production of soluble (undisplayed) protein for more in-depth analysis. For most monovalent display vectors this is facilitated by the presence of an amber stop codon between the displayed protein and pIII, allowing soluble non-fusion protein to be expressed alone by transfer to a non-suppressor strain of *E. coli* (45, 60) (see *Figure 5*). Often the expression and subsequent binding analysis can be performed at small scale in 96-well plates (e.g. see Chapter 13). For shorter peptide sequences, expression as fusions to well-characterized affinity proteins such as maltose binding protein (MBP) can allow confirmation of the properties observed for the displayed peptide (see Chapter 6).

With efficient procedures available for library generation and selection, downstream analysis of clones can frequently be the rate-limiting step in a phage display project. This is leading to the increasing use of automation in an effort to improve throughput: for example, the use of Q-pix and Q-bot robots for colony picking is described in Chapter 11.

Common problems

Many chapters in this book contain troubleshooting hints for specific aspects of phage display, or for particular applications: for example, a guide for phage display binding selections is provided in Chapter 4 (*Table 3*). However, there are several more general potential pitfalls and problems to avoid that can be identified.

Library quality

A key principle of phage display is that the ability to select clones with desired properties is highly dependent on the quality of the initial library—a library that is of insufficient size, poor or inappropriate diversity, or flawed design is unlikely

to lead to success. It is worth investing the time to ensure and then confirm the quality and diversity of the starting library—for example, by using a template encoding an inactive protein when creating a library using oligonucleotide-directed mutagenesis (see Chapter 2, Section 3.3).

Expression editing

An assumption made when a diversified library is created for phage display is that all clones will display with similar efficiency. In fact, some sequences will be refractory to display and therefore underrepresented in the displayed library—in the extreme, the optimal clone (e.g. the one with highest affinity) may never be isolated because it fails to display. For example, in peptide libraries, cysteine residues are often rarer than expected based on predictions of randomness. This has been attributed to reduced display due to formation of inappropriate disulfide bonds (see Chapter 7, Section 2.2). Other sequences may be toxic to *E. coli* or interfere with phage assembly, or be sensitive to bacterial proteases. This "expression editing" means that one cannot assume that every clone theoretically contained within a library has indeed been surveyed in a binding selection.

Over-selection

A common observation in monovalent display, but one that understandably often fails to appear in publications, is that apparently successful selections can often yield clones with strange and unexpected structures, or those that upon analysis do not have the property selected for (e.g. higher affinity). In many cases these outcomes can be attributed to over-selection of the library beyond the rounds at which the desired clones are dominant. Under these conditions, the imposed selective pressure for binding affinity (for example) becomes ineffective, since nearly all clones at that stage will be of equivalent affinity, and factors such as expression level and valency start to drive selection. The result is often that bizarre clones are selected—for example those with internal duplications that lead to bivalency, or those of weak affinity but which are displayed at very high levels. Such observations are a testimony to the power of selection techniques, but emphasize that selections should be carefully monitored, and samples from each round of selection preserved for future analysis.

Alternative display systems

A number of variations on classical filamentous phage display have been described. These are not generally covered in this book, although many of the principles and protocols may be applicable. Fos–Jun fusion display was developed as an early and clever solution to the problem of how to display proteins C-terminally (84). The Jun leucine zipper sequence is displayed conventionally on pIII; a second protein is co-secreted that comprises the Fos leucine zipper with the displayed protein fused at its C-terminus. Fos–Jun interaction and subsequent

disulfide formation provides a C-terminally displayed protein system that has been useful for cDNA interaction cloning. A second technique, termed selectively-infective phage (SIP), exploits the modularity of pIII (see *Figure 3*) to establish a "two-hybrid"-related system in which pIII is split into two pieces, rendering the phage noninfectious. A high-affinity interaction between proteins fused to the pIII domains restore infectivity, allowing identification of binding partners without the need for affinity selection (85).

In addition, bacteriophages other than the Ff family have been used for display of polypeptides, including the lambda (86), T4 (87), and T7 phages (e.g. see (88)). Display as C-terminal fusions to the gene 10 capsid protein of phage T7, in particular, is being increasingly used due to the availability of commercial kits and libraries (see Section 8). Because these phage are lytic, in many cases display does not involve the secretion of the fusion proteins, potentially conferring an advantage for display of some normally intracellular proteins (although see Section 4.1). An application of T7 display phage is described in Chapter 10. Lytic phage are also useful for cDNA display (see Chapter 12, Section 2.2).

Finally, alternative platforms for *in vitro* selection outside of phage display include display on ribosomes following arrest of translation, and display on DNA binding proteins such as the lac repressor (for a review, see (89)). In some cases these techniques have been used together with phage display in a single project aimed at discovering and then improving new binding molecules (see Chapter 6).

Commercial sources of phage display libraries and kits

A number of vectors, libraries, and complete systems for phage display are now available commercially, as indicated in *Table 4*. New England Biolabs supplies a series of pre-made polyvalent pIII libraries displaying linear or disulfide-constrained peptides, and these are a sensible and efficient starting point for investigators searching for simple peptide ligands. Libraries are provided in the form of kits that include appropriate *E. coli* strains, control reagents, sequencing primers, and protocols. A starting vector is also available for construction of custom polyvalent display libraries.

Amersham Biosciences provides the RPAS system for creation of libraries of single-chain Fv antibody fragments displayed monovalently on pIII (using similar principles and protocols to those outlined in Chapter 13). Premade libraries are not supplied, but a series of modules is available for constructing libraries, performing selections, and expressing the selected antibodies. The RPAS system also represents a commercial source for a pIII phagemid display vector for the construction of custom monovalent display libraries.

Novagen commercializes a series of kits for the creation, packaging, and selection of libraries for display on bacteriophage T7. Several pre-made cDNA display libraries are also available, as well as starting vectors for the creation of custom libraries displayed at a range of valencies.

Table 4 Commercial suppliers of phage display vectors and libraries

Manufacturer	Tradename	Vector	Reagents available	Website
New England Biolabs	PhD™ phage display system	M13KE pIII phage vector	Pre-made peptide libraries with control target and eluant; starting phage vector	www.neb.com
Amersham Biosciences	Recombinant Phage Antibody System (RPAS)	pCANTAB-5E pIII phagemid vector	Kits for library construction, selections, phage enzyme-linked immunosorbant assays (ELISAs), and expression of soluble proteins; starting phagemid vector	www.amershambiosciences.com
Novagen	T7Select® System	T7Select vectors: for C-terminal display on gene 10 capsid protein of T7 phage	Kits for library construction, phage *in vitro* packaging, and selections; pre-made cDNA display libraries	www.novagen.com

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